ARGININE REPRESSOR DEFICIENT STRAIN OF CORYNEFORM

BACTERIUM AND METHOD FOR PRODUCING L-ARGININE

Field of the Invention

5   The present invention relates to a coryneform

bacterium having an ability to produce L-arginine and a

method for producing L-arginine using the bacterium. L-

arginine is an industrially useful amino acid as an

ingredient of liver function promoting agents, amino

10   acid infusions, comprehensive amino acid pharmaceuticals

and so forth.

Description of the Related Art

      Conventional L-arginine production by

15   fermentation has been performed by utilizing wild-type

strains of coryneform bacteria; coryneform bacteria

resistant to certain agents including sulfa drugs, 2-

thiazolealanine, á-amino-á-hydroxyvaleric acid and the

like; coryneform bacteria exhibiting auxotrophy for L-

20   histidine, L-proline, L-threonine, L-isoleucine, L-

methionine, or L-tryptophan in addition to the

resistance to 2-thiazolealanine (Japanese Patent Laid-

open No. 54-44096); coryneform bacteria resistant to

ketomalonic acid, fluoromalonic acid, or

25   monofluoroacetic acid (Japanese Patent Laid-open No. 57-

18989); coryneform bacteria resistant to argininol

(Japanese Patent Laid-open No. 62-24075); coryneform

bacteria resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995) or the like.

On the other hand, there have also been disclosed
5   methods for producin g L-arginine utilizing recombinant DNA techniques. That is, there has been disclosed a method for producing L-arginine by utilizing a microorganism belonging to the genus *Corynebacterium* or *Brevibacterium* which is made to harbor a recombinant DNA
10   comprising a vector DNA and a DNA fragment containing genes for acetylornithine deacetylase, N-acetylglutamic acid-ā-semialdehyde dehydrogenase, N-acetyl glutamokinase, and argininosuccinase derived from a microorganism belonging to the genus *Escherichia*
15   (Japanese Patent Publication No. 5-23750).

Further, as for coryneform bacteria, it has been elucidated that synthesis of some enzymes of the L-arginine biosynthetic system is repressed by L-arginine. Furthermore, it was reported that, while some of enzymes
20   of L-arginine biosynthetic system were repressed by L-arginine, the repression of these enzymes by L-arginine was canceled in mutant strains of coryneform bacteria showing improved L-arginine accumulation amounts (*Agric. Biol. Chem.*, 43(1), 105, 1979).

25   Meanwhile, as for *Escherichia coli*, a repressor of L-arginine biosynthetic system and a gene coding for the repressor were identified (*Proc. Natl. Acad. Sci. U.S.A.*

(1987), *84*(19), 6697-701), and binding interactions of
the repressor protein and various genes of L-arginine
biosynthetic system were also investigated (*Proc. Natl.
Acad. Sci. U.S.A.* (1987), *84*(19), 6697-701, *J. Mol. Biol.*
(1992), *226*, 367-386).

However, any repressor proteins of the L-arginine
biosynthetic system have not been identified in
coryneform bacteria. While a nucleotide sequence of the
repressor protein gene (*argR*) and an amino acid sequence
assumed to be encoded thereby are registered in a gene
database, GenBank (AF049897), the gene is considered to
be designated *argR* because of the homology between the
aforementioned amino acid sequence and known arginine
repressors.

Summary of the Invention

As described above, although a repressor protein
of the L-arginine biosynthetic system of coryneform
bacteria and a gene thereof are expected, the repressor
protein itself has not been identified and its functions
and so forth are not elucidated at all. Therefore, an
object of the present invention is to identify the
repressor of the L-arginine biosynthesis in coryneform
bacteria, and improve L-arginine productivity of
coryneform bacteria.

The inventors of the present invention isolated a

homologue of the gene registered as *argR* in the gene database (GenBank accession AF049897) from a coryneform bacterium, and found that, if this gene was amplified in coryneform bacteria, L-arginine producing ability was decreased, and on the other hand, if the gene was disrupted, the L-arginine producing ability was improved, to confirm that the L-arginine biosynthesis is repressed by a repressor in coryneform bacteria like *Escherichia coli* and the aforementioned gene registered as *argR* codes for the repressor. Thus, the present invention was accomplished.

That is, the present invention provides the followings.

(1) A coryneform bacterium in which an arginine repressor does not function in a normal manner, and which has L-arginine producing ability.

(2) The coryneform bacterium according to (1), wherein the arginine repressor does not function in a normal manner due to disruption of a gene coding for the arginine repressor on a chromosome of the bacterium.

(3) The coryneform bacterium according to (2), wherein the arginine repressor has the amino acid sequence shown in SEQ ID NO: 18 or an amino acid sequence showing homology to the amino acid sequence.

(4) A method for producing L-arginine, which comprises culturing a coryneform bacterium according to any one of (1) to (3) in a medium to produce and accumulate L-

arginine in the medium, and collecting the L-arginine from the medium.

In the present invention, the "arginine repressor" refers to a protein that has an effect of repressing the L-arginine biosynthesis, and if expression amount of the gene that codes for the protein increases in coryneform bacteria, L-arginine producing ability will be reduced, and if the expression amount decreases or the protein disappears, the L-arginine producing ability will be improved. Hereafter, the gene coding for the arginine repressor is also called *argR* gene. Further, the "L-arginine producing ability" used in the present invention refers to an ability of the microorganism of the present invention to accumulate L-arginine in a medium, when it is cultured in the medium.

According to the present invention, L-arginine producing ability of coryneform bacteria having the L-arginine producing ability can be improved.

Brief Explanation of the Drawings

Fig. 1 shows the construction process of plasmid pK1.

Fig. 2 shows the construction process of plasmid pSFK6.

Fig. 3 shows the construction process of plasmid pSFKT2.

Hereafter, the present invention will be explained in detail.

The microorganism of the present invention is a coryneform bacterium having L-arginine producing ability, in which arginine repressor does not function in a normal manner. The coryneform bacterium of the present invention may be a microorganism having the L-arginine producing ability because an arginine repressor does not function in a normal manner in it, or a microorganism bred so that the arginine repressor should not function in a normal manner in it. Alternatively, it may be a microorganism that is bred so that the arginine repressor should not function in a normal manner in it and then imparted with the L-arginine producing ability.

The coryneform bacteria include bacteria having been hitherto classified into the genus *Brevibacterium* but united into the genus *Corynebacterium* at present (*Int. J. Syst. Bacteriol.*, *41*, 255 (1981)), and include bacteria belonging to the genus *Brevibacterium* closely relative to the genus *Corynebacterium*. Examples of such coryneform bacteria include the followings.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium alkanolyticum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (Corynebacterium glutamicum)

Corynebacterium melassecola

Corynebacterium thermoaminogenes

Corynebacterium herculis

Brevibacterium divaricatum (Corynebacterium glutamicum)

Brevibacterium flavum (Corynebacterium glutamicum)

Brevibacterium immariophilum

Brevibacterium lactofermentum (Corynebacterium glutamicum)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium album

Brevibacterium cerinum

Microbacterium ammoniaphilum

While the coryneform bacteria that have the L-arginine-producing ability are not particularly limited so long as they have the L-arginine-producing ability, they include, for example, wild-type strains of coryneform bacteria; coryneform bacteria resistant to certain agents including sulfa drugs, 2-thiazolealanine, α-amino-β-hydroxyvaleric acid and the like; coryneform bacteria exhibiting auxotrophy for L-histidine, L-proline, L-threonine, L-isoleucine, L-methionine, or L-tryptophan in addition to the resistance to 2-

thiazolealanine (Japanese Patent Laid-open No. 54-44096); coryneform bacteria resistant to ketomalonic acid, fluoromalonic acid, or monofluoroacetic acid (Japanese Patent Laid-open No. 57-18989); coryneform

5 bacteria resistant to argininol (Japanese Patent Laid-open No. 62-24075); coryneform bacteria resistant to X-guanidine (X represents a derivative of fatty acid or aliphatic chain, Japanese Patent Laid-open No. 2-186995) and the like.

10   Specifically, the following strains can be exemplified.

    Brevibacterium flavum AJ11169 (BP-6892)

    Corynebacterium glutamicum AJ12092 (FERM BP-6906)

    Brevibacterium flavum AJ11336 (FERM BP-6893)

15   *Brevibacterium flavum* AJ11345 (FERM BP-6894)

    Corynebacterium glutamicum AJ12430 (FERM BP-2228)

    The AJ11169 strain and the AJ12092 strain are the 2-thiazolealanine resistant strains mentioned in Japanese Patent Laid-open No. 54-44096, the AJ11336

20 strain is the strain having argininol resistance and sulfadiazine resistance mentioned in Japanese Patent Publication No. 62-24075, the AJ11345 strain is the strain having argininol resistance, 2-thiazolealanine resistance, sulfaguanidine resistance, and exhibiting

25 histidine auxotrophy mentioned in Japanese Patent Publication No. 62-24075, and the AJ12430 strain is the strain having octylguanidine resistance and 2-

thiazolealanine resistance mentioned in Japanese Patent
Laid-open No. 2-186995.

AJ11169 was deposited on August 3, 1977 in
National Institute of Bioscience and Human Technology,
Agency of Industrial Science and Technology, Ministry of
International Trade and Industry (currently National
Institute of Bioscience and Human Technology, National
Institute of Advanced Industrial Science and Technology,
Ministry of Economy, Trade and Industry)(zip code: 305-
8566, 1-3 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken,
Japan), as deposition number of FERM P-4161, and
transferred from the original deposit to international
deposit based on Budapest Treaty on September 27, 1999,
and has been deposited as deposition number of FERM BP-
6892.

AJ12092 was deposited on September 29, 1983 in
National Institute of Bioscience and Human Technology,
Agency of Industrial Science and Technology, Ministry of
International Trade and Industry, as deposition number
of FERM P-7273, and transferred from the original
deposit to international deposit based on Budapest
Treaty on October 1, 1999, and has been deposited as
deposition number of FERM BP-6906.

AJ11336 was deposited on April 25, 1979 in
National Institute of Bioscience and Human Technology,
Agency of Industrial Science and Technology, Ministry of
International Trade and Industry, as deposition number

of FERM P-4939, and transferred from the original
deposit to international deposit based on Budapest
Treaty on September 27, 1999, and has been deposited as
deposition number of FERM BP-6893.

5   AJ11345 was deposited on April 25, 1979 in
National Institute of Bioscience and Human Technology,
Agency of Industrial Science and Technology, Ministry of
International Trade and Industry, as deposition number
of FERM P-4948, and transferred from the original

10   deposit to international deposit based on Budapest
Treaty on September 27, 1999, and has been deposited as
deposition number of FERM BP-6894.

       AJ12430 was deposited on December 26, 1988 in
National Institute of Bioscience and Human Technology,

15   Agency of Industrial Science and Technology of Ministry,
International Trade and Industry based on Budapest
Treaty, as deposition number of FERM BP-2228.

       The coryneform bacterium whose arginine repressor
does not function in a normal manner can be obtained by

20   modifying its argR gene so that the activity of the
arginine repressor should be reduced or eliminated, or
the transcription of the argR gene should be reduced or
eliminated. Such a coryneform bacterium can be obtained
by, for example, replacing the chromosomal argR gene

25   with an argR gene that does not function in a normal
manner (occasionally referred to as "disrupted argR
gene" hereinafter) through, for example, homologous

recombination based on genetic recombination techniques
(Experiments in Molecular Genetics, Cold Spring Harbor
Laboratory Press (1972); Matsuyama, S. and Mizushima, S.,
J. Bacteriol., *162*, 1196 (1985)).

In the homologous recombination, when a plasmid
carrying a sequence exhibiting homology with a
chromosomal sequence or the like is introduced into a
corresponding bacterial cell, recombination occurs at a
site of the homologous sequence at a certain frequency,
and thus the introduced plasmid as a whole is integrated
into the chromosome. Then, by causing recombination
again at the site of the homologous sequence in the
chromosome, the plasmid may be removed from the
chromosome. However, depending on the position at which
the recombination is caused, the disrupted gene may
remain on the chromosome, while the original normal gene
may be removed from the chromosome together with the
plasmid. By selecting such a bacterial strain, a
bacterial strain in which the normal *argR* gene is
replaced with a disrupted *argR* gene can be obtained.

Such a gene disruption technique based on the
homologous recombination has already been established,
and a method utilizing a linear DNA, method utilizing
temperature sensitive plasmid or the like can be used
therefor. The *argR* gene can also be disrupted by using
a plasmid that contains the *argR* gene inserted with a
marker gene such as drug resistance gene, and cannot

replicate in a target cell of the coryneform bacterium.
That is, in a transformant that has been transformed
with such a plasmid and hence acquired drug resistance,
the marker gene is integrated in a chromosome DNA. It

5   is likely that this marker gene has been integrated by
homologous recombination of the *argR* gene present at the
both sides of the marker with the *argR* on the chromosome,
and therefore a gene-disrupted strain can efficiently be
selected.

10   Specifically, a disrupted *argR* gene used for the
gene disruption can be obtained by deletion of a certain
region of *argR* gene by means of digestion with
restriction exzyme(s) and religation; by insertion of
another DNA fragment (marker gene etc.) into the *argR*

15   gene, by introducing substitution, deletion, insertion,
addition or inversion of one or more nucleotides in a
nucleotide sequence of coding region of *argR* gene, its
promoter region or the like by means of site-specific
mutagenesis (Kramer, W. and Frits, H. J., Methods in

20   Enzymology, *154*, 350 (1987)) or treatment with a
chemical reagent such as sodium hyposulfite and
hydroxylamine (Shortle, D. and Nathans, D., *Proc. Natl.
Acad. Sci. U.S.A.*, *75*, 270(1978)) or the like, so that
the activity of the encoded repressor should be reduced

25   or eliminated, or transcription of the *argR* gene should
be reduced or eliminated. Among these embodiments, a
method utilizing deletion of a certain region of the

argR gene by digestion with a restriction enzyme and religation, or insertion of another DNA fragment into the *argR* gene is preferred in view of reliability and stability.

5   A plasmid for the *argR* gene disruption can be produced by performing PCR (polymerase chain reaction) using a plasmid containing the *argR* gene and its flanking regions as a template and primers corresponding the terminal portions or franking regions of the *argR*

10   gene to amplify a portion except for an internal portion or the whole portion of the *argR* gene, and cyclizing the obtained amplified product. In the examples mentioned hereinafter, the *argR* gene was disrupted by this method.

The *argR* gene can be obtained from a chromosomal

15   DNA of a coryneform bacterium by PCR using oligonucleotides prepared based on known nucleotide sequences of the *argR* gene as primers. The *argR* gene can also be obtained from a chromosome DNA library of a microorganism which has a purine operon by a

20   hybridization technique using an oligonucleotide prepared based on a known nucleotide sequence of the *argR* gene as a probe. For the purpose of the present invention, because the *argR* gene is used for preparing a disrupted *argR* gene, it is not necessarily required to

25   contain the full length, and it may contain a length required to cause gene disruption.

The origin of the *argR* gene is not particularly

limited, so long as it has such a degree of homology
that it should cause homologous recombination with the
argR gene of coryneform bacteria. Specifically, the
argR gene of the *Brevibacterium flavum*, which has the
nucleotide sequence shown in SEQ ID NO: 17, and the *argR*
gene of *Corynebacterium glutamicum* (GenBank accession
AF049897) can be mentioned as the *argR* genes of
coryneform bacteria. These *argR* genes are highly
homologous, and it is considered that even an *argR* gene
of coryneform bacterium of a genus or species different
from that of a coryneform bacterium of which *argR* gene
is to be disrupted may also be used for the gene
disruption.

In the present invention, the amino acid sequence
shown in SEQ ID NO: 18 or an amino acid sequence
exhibiting homology to the amino acid sequence means an
amino acid sequence that is encoded by an *argR* gene
having such a degree of homology that it should cause
homologous recombination with the *argR* gene coding to
the amino acid sequence shown in SEQ ID NO: 18 (for
example, an *argR* gene having the nucleotide sequence
shown in SEQ ID NO: 17).

As the primers used for PCR, any primers that
allow amplification of the *argR* gene can be used.
Specific examples thereof include the oligonucleotides
having the nucleotide sequences shown in SEQ ID NOS: 19
and 20.

Further, examples of marker gene include drug resistance genes such as a kanamycin resistance gene. A kanamycin resistance gene can be obtained by PCR amplification from a known plasmid containing a kanamycin resistance gene of *Streptococcus faecalis*, for example, pDG783 (Anne-Marie Guerout-Fleury *et al.*, *Gene*, *167*, 335-337 (1995)).

When a drug resistance gene is used as the marker gene, an *argR* gene-disrupted strain can be obtained by inserting the drug resistance gene into a suitable site of the *argR* gene carried by a plasmid, transforming a microorganism with the plasmid, and selecting a drug resistant transformant. Disruption of *argR* gene on a chromosome can be confirmed by analyzing the *argR* gene or the marker gene on the chromosome by Southern blotting, PCR, or the like. Integration of the kanamycin resistance gene into a chromosomal DNA can be confirmed by PCR using primers that allow amplification of the kanamycin resistance gene (e.g., oligonucleotides having nucleotide sequences shown in SEQ ID NOS: 1 and 2).

L-arginine can be efficiently produced by culturing a coryneform bacterium having L-arginine producing ability obtained as described above, in which an arginine repressor does not function in a normal manner, in a medium to produce and accumulate L-arginine in the medium, and collecting the L-arginine from the

medium.

The medium to be used may be selected from well-known media conventionally used for fermentative production of amino acids utilizing microorganisms.

5   That is, it may be a usual medium that contains a carbon source, nitrogen source, inorganic ions, and other organic ingredients as required.

As the carbon source, there can be used saccharides such as glucose, sucrose, lactose, galactose,

10 fructose or starch hydrolysate, alcohols such as glycerol or sorbitol, or organic acids such as fumaric acid, citric acid or succinic acid.

As the nitrogen source, there can be used inorganic ammonium salts such as ammonium sulfate,

15 ammonium chloride or ammonium phosphate, organic nitrogen such as soybean protein hydrolysate, ammonia gas, aqueous ammonia and so forth.

It is desirable to add required substances such as vitamin B_1 and L-homoserine, yeast extract and so forth

20 to the medium in appropriate amounts as organic trace nutrients. Other than the above, potassium phosphate, magnesium sulfate, iron ion, manganese ion and so forth are added in small amounts as required.

The culture is preferably carried out under an

25 aerobic condition for 1-7 days. The culture temperature is preferably controlled to be 24°C to 37°C, and pH is preferably controlled to be 5-9 during the culture.

Inorganic or organic, acidic, alkaline substances, or ammonia gas and so forth can be used for pH adjustment. L-arginine can be collected from the fermentation broth usually by a combination of well-known techniques such as ion exchange resin techniques and other techniques.

Best Mode for Carrying out the Invention

Hereafter, the present invention will be explained more specifically with reference to the following examples.

Example 1: Constructions of shuttle vector for Escherichia coli and coryneform bacteria and temperature sensitive vector

First, a vector for introducing an *argR* gene into coryneform bacteria and a temperature sensitive vector for producing an *argR* deficient strain of coryneform bacterium were constructed.

<1> Construction of vector having drug resistance gene of *Streptococcus faecalis*

The kanamycin resistance gene of *Streptococcus faecalis* was amplified by PCR from a known plasmid containing the gene. The nucleotide sequence of the kanamycin resistance gene of *Streptococcus faecalis* has

already been elucidated (Trieu-Cuot, P. and Courvalin,
P.: *Gene*, *23* (3), 331-341 (1983)). Based on this
sequence, the primers shown in SEQ ID NOS: 1 and 2 were
synthesized, and PCR was performed by using pDG783

5   (Anne-Marie Guerout-Fleury, *et al.*, *Gene*, *167*, 335-337
(1995)) as a template to amplify a DNA fragment
containing the kanamycin resistance gene and its
promoter.

The aforementioned DNA fragment was purified by

10 using SUPREC02 produced by Takara Shuzo Co., Ltd., and
then completely digested with restriction enzymes
*Hind*III and *Hinc*II to be blunt-ended. The blunt-ending
was performed by using Blunting Kit produced by Takara
Shuzo Co., Ltd. This DNA fragment was mixed with a DNA

15 fragment obtained by purification and blunt-ending of an
amplification product of PCR performed by using the
primers shown in SEQ ID NOS: 3 and 4 and pHSG399 (see S.
Takeshita, *et al.*: *Gene*, *61*, 63-74 (1987)) as a template,
and ligated both fragments. The ligation was performed

20 by using DNA Ligation Kit Ver. 2 produced by Takara
Shuzo Co., Ltd. Competent cells of *Escherichia coli*
JM109 (produced by Takara Shuzo Co., Ltd.) were
transformed with the ligated DNA, plated on L medium (10
g/L of Bacto trypton, 5 g/L of Bacto yeast extract, 5

25 g/L of NaCl, 15 g/L of agar, pH 7.2) containing 10 μg/ml
of IPTG (isopropyl-â-D-thiogalactopyranoside), 40 μg/ml
of X-Gal (5-bromo-4-chloro-3-indolyl-â-D-galactoside)

and 25 μg/ml of kanamycin, and cultured overnight. The

emerged blue colonies were picked up, and separated into

single colonies to obtain transformant strains.

     Plasmids were prepared from the transformant

5   strains by the alkali method (Seibutsu Kogaku Jikkensyo

(Text for Bioengineering Experiments), Edited by the

Society for Bioscience and Bioengineering, Japan, p.105,

Baifukan, 1992), and restriction maps were prepared.

One having a restriction map equivalent to that of Fig.

10   1 was designated as pK1. This plasmid is stably

harbored in *Escherichia coli*, and imparts kanamycin

resistance to a host. Moreover, since it contains the

lacZ' gene, it is suitably used as a cloning vector.

15   <2> Construction of shuttle vector pSFK6

     As a material for obtaining a temperature

sensitive replication control region, a plasmid vector

autonomously replicable in both of *Escherichia coli*

cells and coryneform bacteria cells was prepared. The

20   plasmid pAM330 extracted from Brevibacterium

lactofermentum ATCC13869 [see Japanese Patent

Publication Laid-open (Kokai) No. 58-67699] was

completely digested with a restriction enzyme *Hind*III,

and blunt-ended. This fragment was ligated to a

25   fragment obtained by completely digesting the

aforementioned pK1 with a restriction enzyme *Bsa*AI.

Brevibacterium lactofermentum ATCC13869 was transformed

with the ligated DNA. The transformation was performed
by the electric pulse method [see Japanese Patent
Publication Laid-open (Kokai) No. 2-207791].

Transformants were selected on an M-CM2B plate (10 g/L
5   of polypeptone, 10 g/L of yeast extract, 5 g/L of NaCl,
10 μg/L of biotin, 15 g/L of agar, pH 7.2) containing
25 μg/ml of kanamycin. After cultivation for 2 days,
colonies were picked up, and separated into single
colonies to obtain the transformants. Plasmid DNAs were
10   prepared from the transformants, and restriction maps
were prepared. One having the same restriction map as
that of Fig. 2 was designated as pSFK6. This plasmid is
autonomously replicable in both of *Escherichia coli* and
coryneform bacteria, and imparts kanamycin resistance to
15   a host.

<3> Construction of a plasmid having temperature
sensitive replication control region

     pSFK6 was treated with hydroxylamine *in vitro*.
20   The hydroxylamine treatment was performed according to a
known method [see, for example, G.O. Humpherys, *et al.*,
Molec. Gen. Genet., *145*, 101-108 (1976)]. DNA undergone
the treatment was collected and used for transformation
of Brevibacterium lactofermentum ATCC13869 strain. The
25   transformants were selected at a low temperature (25°C)
on a CM2B plate containing 25 μg/ml of kanamycin. The
appeared transformants were replicated to a similar

selection plate, and cultured at an elevated temperature (34°C). One strain that could not grow on the selection plate containing kanamycin at the elevated temperature was obtained. From this strain, a plasmid was recovered

5   and designated as p48K.

<4> Determination of nucleotide sequence of temperature sensitive replication control region

Nucleotide sequences of replication control region

10   segments in the plasmid pSFK6 having a wild-type replication control region and the plasmid p48K having a temperature sensitive replication control region were determined. The nucleotide sequences were determined on a fully automatic sequencer, ABI310 (ABI), by using DNA

15   Sequencing Kit from ABI. As a result, it was found that there were 6 nucleotide substitutions between the wild-type replication control region and the temperature sensitive replication control region. The nucleotide sequence of the temperature sensitive replication control region segment contained in pSFK6 (derived from

20   full sequence of pAM330), which functions in coryneform bacteria, is shown in SEQ ID NO: 5, and the nucleotide sequence of the temperature sensitive replication control region segment contained in p48K, which

25   functions in coryneform bacteria, is shown in SEQ ID NO: 7. Further, the amino acid sequences encoded by ORFs contained in these nucleotide sequences are shown in SEQ

ID NOS: 6 and 8. In the temperature sensitive replication control region, the 1255th C is mutated to T, the 1534th C to T, the 1866th G to A, the 2058th G to A, the 2187th C to T and 3193rd G to A. Among these, only the mutation at 1534th position is accompanied by an amino acid mutation, and causes substitution of serine for proline.

<5> Construction of shuttle vectors having temperature sensitive mutation

Each one of the six mutations of p48K was introduced into a shuttle vector pSFK6 (see Fig. 3). The introduction of the mutations was performed by a known method [Mikaelian, I., Sergeant, A., *Nucleic Acids Res.*, *20*, 376 (1992)]. Specific procedure will be mentioned below. In order to introduce the mutation of 1534th C to T, PCR was performed by using a combination of the primers shown in SEQ ID NOS: 9 and 10 (primers 9 and 10), and a combination of the primers shown in SEQ ID NOS: 11 and 12 (primers 11 and 12), and pAM330 as a template. Each of the obtained amplification products was purified by subjecting them to agarose gel electrophoresis, and collecting them from the gel. The collection of the DNA fragments from the gel was performed by using EASYTRAP Ver.2 (Takara Shuzo Co., Ltd.). The purified DNAs were mixed in a molar ratio of 1:1, and used as a template for PCR performed by using

the primers shown SEQ ID NOS: 13 and 14 (primers 13 and
14). The amplification product was fully digested with
a restriction enzyme MluI, and subjected to agarose gel
electrophoresis to recover a DNA fragment of about 3.2

5   kb. Similarly, pSFK6 was also completely digested with
a restriction enzyme MluI, and subjected to agarose gel
electrophoresis to recover a DNA fragment of about 3.8
kb. The obtained DNA fragments were mixed and ligated,
and used to transform competent cells of *Escherichia*

10   *coli* JM109 (Takara Shuzo Co., Ltd.). The cells were
applied on L medium containing 25 μg/ml of kanamycin,
and cultured overnight. The appeared colonies were
picked up, and isolated single colonies to obtain
transformant strains. A plasmid was prepared from the

15   transformant strains by the alkaline method, and the
nucleotide sequence of the plasmid was determined to
confirm that 1534th C in the sequence shown in SEQ ID
NO: 5 was mutated to T. This plasmid was designated as
pSFKT2 (Fig. 3).

20

Example 2: Cloning of *argR* gene and amplification effect
thereof in coryneform bacteria

         PCR was performed by using chromosome DNA of the
25   *Brevibacterium flavum* wild strain 2247 (AJ14067) as a
template and the oligonucleotides having the nucleotide
sequences shown in SEQ ID NO: 15 (sequence of the

nucleotide numbers 1717-1741 in SEQ ID NO: 17) and SEQ

ID NO: 16 (sequence complementary to the sequence of the

nucleotide numbers 2386-2362 in SEQ ID NO: 17) as

primers (Primers 15 and 16). PCR was performed for 30

5   cycles with each cycle consisting of reactions at 98°C

for 10 seconds, 58°C for 1 minute and 72°C for 3 minutes

by using Pyrobest DNA polymerase (Takara Shuzo Co.,

Ltd.). The obtained amplified fragment was inserted

into the *Sma*I site of the shuttle vector pSFK6 obtained

10   in Example 1 to obtain plasmid pWR autonomously

replicable in coryneform bacteria.

     In order to investigate the amplification effect

of *argR* gene in L-arginine producing coryneform bacteria,

pWR was introduced into the AJ113455 strain (FERM BP-

15   6894), which is an L-arginine producer of *Brevibacterium*

flavum. The plasmid was introduced by the electric

pulse method (Japanese Patent Laid-open No. 2-207791).

A transformant was selected as a kanamycin resistant

strain on CM2G agar medium (containing 5 g of glucose, 5

20   g of NaCl and 15 g of agar in 1 L of pure water, pH 7.2)

containing 25 µg/ml of kanamycin to obtain AJ11345/pWR.

As a control, pSFK6 was similarly introduced into the

AJ113455 strain to obtain a transformant AJ11345/pSFK6.

     Each of the aforementioned strains was plated on

25   an agar medium containing 0.5 g/dl of glucose, 1 g/dl of

polypeptone, 1 g/dl of yeast extract and 0.5 g/dl of

NaCl, and cultured at 31.5°C for 20 hours. One platunum

loop of the obtained cells were inoculated into a medium
containing 4 g/dl of glucose, 6.5 g/dl of ammonium
sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001
g/dl of $FeSO_4$, 0.001 g/dl of $MnSO_4$, 5 μg/dl of vitamin B_1,

5   5 μg/dl of biotin and soybean protein hydrolysate (45
mg/dl as N amount), and cultured in a flask at 31.5°C
for 50 hours with shaking. Accumulation amount of L-
arginine (concentration, g/dl) in each culture broth was
measured. The results are shown in Table 1. As a

10   result, the *argR*-amplified strain hardly accumulated L-
arginine. This demonstrated that the *argR* gene product
functioned as an arginine repressor.

Table 1

Strain	L-Arginine accumulation amount (g/dl)
AJ11345/pSFK6	1.3
AJ11345/pWR	0.2

15

The result of nucleotide sequencing for the
inserted fragment cloned in pWR is shown in SEQ ID NO:
17. An amino acid sequence that may be encoded by that
nucleotide sequence is shown in SEQ ID NO: 18.

20

Example 3: Construction of *argR*-disrupted strain of
coryneform bacterium and effect of deletion of arginine
repressor

<1> Construction of plasmid for *argR* disruption

25   PCR was performed by using chromosome DNA of a

wild strain of *Brevibacterium flavum*, 2247 strain (AJ14067), as a template and the oligonucleotides having the nucleotide sequences shown in SEQ ID NO: 19 (sequence of the nucleotide numbers 4-28 in SEQ ID NO: 17) and SEQ ID NO: 20 (sequence complementary to the sequence of the nucleotide numbers 4230-4211 in SEQ ID NO: 17) as primers (Primers 19 and 20). PCR was performed for 30 cycles with each cycle consisting of reactions at 98°C for 10 seconds, 58°C for 1 minute and 72°C for 3 minutes by using Pyrobest DNA polymerase (Takara Shuzo Co., Ltd.). The obtained amplified fragment was inserted into the *Sma*I site in a multicloning site of cloning vector pHSG399.

In order to delete the whole ORF considered to encode the arginine repressor from the inserted DNA fragment, PCR was performed by using the oligonucleotides having the nucleotide sequences shown in SEQ ID NO: 21 (sequence of the nucleotide numbers 2372-2395 in SEQ ID NO: 17) and SEQ ID NO: 22 (sequence complementary to the sequence of the nucleotide numbers 1851-1827 in SEQ ID NO: 17) as primers (Primers 21 and 22) and pHSG399 inserted with the amplified fragment as a template. pssER was constructed by self-ligation of the PCR product.

Then, a fragment obtained by digesting pssER with restriction enzymes *Sma*I and *Sal*I and the temperature sensitive plasmid pSFKT2 obtained in Example 1 and

digested with *Sma*I and *Sal*I were ligated to obtain plasmid pssERT for *argR* disruption whose autonomous replication ability in coryneform bacteria became temperature sensitive.

5

<2> Construction of arginine repressor deficient strain of coryneform bacterium by homologous recombination

The plasmid pssERT obtained as described above was introduced into the *Brevibacterium lactofermentum*

10   AJ13029 strain (FERM BP-5189). The plasmid was introduced by the electric pulse method (Japanese Patent Laid-open No. 2-207791). Because autonomous replication ability of this plasmid is temperature sensitive in *Brevibacterium lactofermentum*, only strains in which

15   this plasmid was incorporated into the chromosome by homologous recombination could be selected as kanamycin resistant strains at 34°C, which was a temperature that did not allow replication of the plasmid. Strains in which the plasmid for *argR* disruption was incorporated

20   into a chromosome were selected as kanamycin resistant strains on a CM2G plate (containing 10 g/L of polypeptone, 10 g/L of yeast extract, 5 g/L of glucose, 5 g/L of NaCl and 15 g/L of agar in 1 L of water, pH 7.2) containing 25 µg/ml of kanamycin. At this stage,

25   the normal *argR* gene derived from the chromosome and the *argR* gene derived from the plasmid in which OFR was deleted were present in tandem at the both sides of the

plasmid portion on the chromosome.

Then, the recombinant strains were allowed to cause homologous recombination again, and strains that became kanamycin sensitive at 34°C, which was a temperature that did not allow the plasmid replication, were selected as strains in which one of the *argR* genes was deleted. These strains include strains in which the normal *argR* gene remained on the chromosome and strains in which the disrupted *argR* gene remained on the chromosome. From these strains, a strain having only the disrupted *argR* gene was selected. An *argR* gene on the chromosome is determined to be the disrupted type by preparing chromosome of a strain that became kanamycin sensitive at 34°C, performing PCR utilizing the chromosome as a template and the oligonucleotides having the nucleotide sequences shown in SEQ ID NOS: 19 and 20 as primers (Primers 19 and 20), and confirming that the PCR product was shorter by about 600 bp than that obtained by similarly performing PCR utilizing chromosome derived from the parent strain as a template.

Direct sequencing of the PCR product of the *argR*-disrupted strain selected as described above was performed to confirm that the *argR* gene was disrupted as desired, and thus AJ13029ΔR stain was obtained.

<3> Production of L-arginine with *argR*-disrupted strain

The AJ13029ΔR strain was plated on an agar medium

containing 0.5 g/dl of glucose, 1 g/dl of polypeptone, 1 g/dl of yeast extract and 0.5 g/dl of NaCl, and cultured at 31.5°C for 20 hours. One platinum loop of the obtained cells were inoculated into a medium containing

5   3 g/dl of glucose, 6.5 g/dl of ammonium sulfate, 0.1 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.001 g/dl of $MnSO_4$, 300 μg/dl of vitamin B_1, 200 μg/dl of biotin and soybean protein hydrolysate (165 mg/dl as N amount) and adjusted to pH 7.0 with NaOH, and cultured

10 at 31.5°C for 24 hours as seed culture.

The above seed culture broth was inoculated in an amount of 1 ml into a medium containing 4 g/dl of glucose, 6.5 g/dl of ammonium sulfate, 0.5 g/dl of KH_2PO_4, 0.04 g/dl of $MgSO_4$, 0.001 g/dl of $FeSO_4$, 0.01

15 g/dl of $MnSO_4$, 5 μg/dl of vitamin B_1, 5 μg/dl of biotin and soybean protein hydrolysate (45 mg/dl as N amount) and adjusted to pH 7.0 with KOH, and cultured in a flask at 31.5°C for 50 hours with shaking. Accumulation amount of L-arginine (concentration, mg/dl) in culture

20 broth of each strain was measured. The results are shown in Table 2. As a result, the argR-disrupted strain accumulated L-arginine in a markedly larger amount compared with the parent strain.

Table 2

Strain	L-Arginine accumulation amount (mg/dl)
AJ13029	20
AJ13029ΔR	120

(Explanation of Sequence Listing)

5 SEQ ID NO: 1: primer for amplification of kanamycin

resistance gene of *Streptococcus faecalis*

SEQ ID NO: 2: primer for amplification of kanamycin

resistance gene of *Streptococcus faecalis*

SEQ ID NO: 3: primer for amplification of vector portion

10 of pHSG399

SEQ ID NO: 4: primer for amplification of vector portion

of pHSG399

SEQ ID NO: 5: nucleotide sequence of replication control

region of pSFK6

15 SEQ ID NO: 6: amino acid sequence that may be encoded by

ORF in pSFK6

SEQ ID NO: 7: nucleotide sequence of replication control

region of p48K

SEQ ID NO: 8: amino acid sequence that may be encoded by

20 ORF in p48K

SEQ ID NO: 9: primer for 1st PCR for introducing

mutation of 1534th C to T into pSFK6

SEQ ID NO: 10: primer for 1st PCR for introducing

mutation of 1534th C to T into pSFK6

SEQ ID NO: 11: primer for 1st PCR for introducing mutation of 1534th C to T into pSFK6

SEQ ID NO: 12: primer for 1st PCR for introducing mutation of 1534th C to T into pSFK6

5   SEQ ID NO: 13: primer for 2nd PCR for introducing mutation of 1534th C to T into pSFK6

SEQ ID NO: 14: primer for 2nd PCR for introducing mutation of 1534th C to T into pSFK6

SEQ ID NO: 15: primer for *argR* gene amplification

10   SEQ ID NO: 16: primer for *argR* gene amplification

SEQ ID NO: 17: nucleotide sequence of DNA fragment containing *argR* gene

SEQ ID NO: 18: amino acid sequence that may be encoded by the above DNA fragment

15   SEQ ID NO: 19: primer for *argR* gene amplification

SEQ ID NO: 20: primer for *argR* gene amplification

SEQ ID NO: 21: primer for amplifying portions other than *argR* gene ORF of plasmid containing *argR* gene

SEQ ID NO: 22: primer for amplifying portions other than

20   *argR* gene ORF of plasmid containing *argR* gene

SEQUENCE LISTING

<110> SUGA, Mikiko
      ASAKURA, Yoko
      MORI, Yukiko
      ITO, Hisao
      KURAHASHI, Osamu

<120> Arginine Repressor Deficient Stain of Coryneform Bacterium and
Method for Producing L-Arginine

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caaacattgt cctgcaactc attcattatg tgcagtgctc ctgttactag tcgtacatac 840
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ggtaagctgg cacgcatagc caagctaggc ggcaccaaa caccactaaa aattaatagt 1140
tcctagacaa gacaaacccc cgtgcgagct accaactcat atgcacgggg gccacataac 1200
ccgaaggggt ttcaattgac aaccatagca ctagctaaga caacgggcac aacatccgca 1260
caaactcgca ctgcgcaacc ccgcacaaca tcgggtctag gtaacactga aatagaa 1317
gtg aac acc tct aag gaa ccg cag gtc aat gag ggt tct aag gtc act 1365
Val Asn Thr Ser Lys Glu Pro Gln Val Asn Glu Gly Ser Lys Val Thr
 1   5   10   15
cgc gct agg gcg tgg cgt agg caa aac gtc atg tac aag atc acc aat 1413
Arg Ala Arg Ala Trp Arg Arg Gln Asn Val Met Tyr Lys Ile Thr Asn
            20   25   30
agt aag gct ctg gcg ggg tgc cat agg tgg cgc agg gac gaa gct gtt 1461
Ser Lys Ala Leu Ala Gly Cys His Arg Trp Arg Arg Asp Glu Ala Val
        35   40   45
gcg gtg tcc tgg tcg tct aac ggt gct tcg cag ttt gag ggt ctg caa 1509
Ala Val Ser Trp Ser Ser Asn Gly Ala Ser Gln Phe Glu Gly Leu Gln
    50   55   60

```
aac tct cac tct cgc tgg ggg tca tct ctg gct gaa ttg gaa gtc atg   1557
Asn Ser His Ser Arg Trp Gly Ser Ser Leu Ala Glu Leu Glu Val Met
 65                  70                  75                  80

ggc gaa cgc cgc att gag ctg gct att gct act aag aat cac ttg gcg   1605
Gly Glu Arg Arg Ile Glu Leu Ala Ile Ala Thr Lys Asn His Leu Ala
                85                  90                  95

gcg ggt ggc gcg ctc atg atg ttt gtg ggc act gtt cga cac aac cgc   1653
Ala Gly Gly Ala Leu Met Met Phe Val Gly Thr Val Arg His Asn Arg
            100                 105                 110

tca cag tca ttt gcg cag gtt gaa gcg ggt att aag act gcg tac tct   1701
Ser Gln Ser Phe Ala Gln Val Glu Ala Gly Ile Lys Thr Ala Tyr Ser
            115                 120                 125

tcg atg gtg aaa aca tct cag tgg aag aaa gaa cgt gca cgg tac ggg   1749
Ser Met Val Lys Thr Ser Gln Trp Lys Lys Glu Arg Ala Arg Tyr Gly
        130                 135                 140

gtg gag cac acc tat agt gac tat gag gtc aca gac tct tgg gcg aac   1797
Val Glu His Thr Tyr Ser Asp Tyr Glu Val Thr Asp Ser Trp Ala Asn
145                 150                 155                 160

ggt tgg cac ttg cac cgc aac atg ctg ttg ttc ttg gat cgt cca ctg   1845
Gly Trp His Leu His Arg Asn Met Leu Leu Phe Leu Asp Arg Pro Leu
                165                 170                 175

tct gac gat gaa ctc aag gca ttt gag gat tcc atg ttt tcc cgc tgg   1893
Ser Asp Asp Glu Leu Lys Ala Phe Glu Asp Ser Met Phe Ser Arg Trp
            180                 185                 190

tct gct ggt gtg gtt aag gcc ggt atg gac gcg cca ctg cgt gag cac   1941
Ser Ala Gly Val Val Lys Ala Gly Met Asp Ala Pro Leu Arg Glu His
            195                 200                 205

ggg gtc aaa ctt gat cag gtg tct acc tgg ggt gga gac gct gcg aaa   1989
Gly Val Lys Leu Asp Gln Val Ser Thr Trp Gly Gly Asp Ala Ala Lys
        210                 215                 220

atg gca acc tac ctc gct aag ggc atg tct cag gaa ctg act ggc tcc   2037
Met Ala Thr Tyr Leu Ala Lys Gly Met Ser Gln Glu Leu Thr Gly Ser
225                 230                 235                 240

gct act aaa acc gcg tct aaa ggg tcg tac acg ccg ttt cag atg ttg   2085
Ala Thr Lys Thr Ala Ser Lys Gly Ser Tyr Thr Pro Phe Gln Met Leu
                245                 250                 255

gat atg ttg gcc gat caa agc gac gcc ggc gag gat atg gac gct gtt   2133
Asp Met Leu Ala Asp Gln Ser Asp Ala Gly Glu Asp Met Asp Ala Val
            260                 265                 270

ttg gtg gct cgg tgg cgt gag tat gag gtt ggt tct aaa aac ctg cgt   2181
Leu Val Ala Arg Trp Arg Glu Tyr Glu Val Gly Ser Lys Asn Leu Arg
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          275                   280                   285
tcg tct tgg tca cgt ggg gct aag cgt gct ttg ggc att gat tac ata   2229
Ser Ser Trp Ser Arg Gly Ala Lys Arg Ala Leu Gly Ile Asp Tyr Ile
      290                   295                   300
gac gct gat gta cgt cgt gaa atg gaa gaa gaa ctg tac aag ctc gcc   2277
Asp Ala Asp Val Arg Arg Glu Met Glu Glu Glu Leu Tyr Lys Leu Ala
305                   310                   315                   320
ggt ctg gaa gca ccg gaa cgg gtc gaa tca acc cgc gtt gct gtt gct   2325
Gly Leu Glu Ala Pro Glu Arg Val Glu Ser Thr Arg Val Ala Val Ala
                  325                   330                   335
ttg gtg aag ccc gat gat tgg aaa ctg att cag tct gat ttc gcg gtt   2373
Leu Val Lys Pro Asp Asp Trp Lys Leu Ile Gln Ser Asp Phe Ala Val
              340                   345                   350
agg cag tac gtt cta gat tgc gtg gtg aag gct aag gac gtg gcc gct   2421
Arg Gln Tyr Val Leu Asp Cys Val Asp Lys Ala Lys Asp Val Ala Ala
          355                   360                   365
gcg caa cgt gtc gct aat gag gtg ctg gca agt ctg ggt gtg gat tcc   2469
Ala Gln Arg Val Ala Asn Glu Val Leu Ala Ser Leu Gly Val Asp Ser
      370                   375                   380
acc ccg tgc atg atc gtt atg gat gat gtg gac ttg gac gcg gtt ctg   2517
Thr Pro Cys Met Ile Val Met Asp Asp Val Asp Leu Asp Ala Val Leu
385                   390                   395                   400
cct act cat ggg gac gct act aag cgt gat ctg aat gcg gcg gtg ttc   2565
Pro Thr His Gly Asp Ala Thr Lys Arg Asp Leu Asn Ala Ala Val Phe
                  405                   410                   415
gcg ggt aat gag cag act att ctt cgc acc cac taaaagcggc ataaaccccg 2618
Ala Gly Asn Glu Gln Thr Ile Leu Arg Thr His
              420                   425
ttcgatattt tgtgcgatga atttatggtc aatgtcgcgg gggcaaacta tgatgggtct  2678
tgttgttgac aatggctgat ttcatcagga atggaactgt catgctgtta tgtgcctggc  2738
tcctaatcaa agctggggac aatgggttgc cccgttgatc tgatctagtt cggattggcg  2798
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cagttttcga gttcactggg attttggtg catcaaacaa gatgtaggac aatgcggttt  3218
ctaggtctac tttttgcttt atgccgtaca agccccgtgg gtattcagcg attgattcca  3278
aggcggcttc ccagtcctgt tttgtgaagg actggcttag ttctaggtct gtgtctgggt  3338
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```

<210> 8
<211> 427
<212> PRT
<213> Brevibacterium lactofermentum

<400> 8

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 1               5                   10                  15
Arg Ala Arg Ala Trp Arg Arg Gln Asn Val Met Tyr Lys Ile Thr Asn
            20                  25                  30
Ser Lys Ala Leu Ala Gly Cys His Arg Trp Arg Arg Asp Glu Ala Val
        35                  40                  45
Ala Val Ser Trp Ser Ser Asn Gly Ala Ser Gln Phe Glu Gly Leu Gln
    50                  55                  60
Asn Ser His Ser Arg Trp Gly Ser Ser Leu Ala Glu Leu Glu Val Met
 65                  70                  75                  80
Gly Glu Arg Arg Ile Glu Leu Ala Ile Ala Thr Lys Asn His Leu Ala
                85                  90                  95
Ala Gly Gly Ala Leu Met Met Phe Val Gly Thr Val Arg His Asn Arg
            100                 105                 110
Ser Gln Ser Phe Ala Gln Val Glu Ala Gly Ile Lys Thr Ala Tyr Ser
        115                 120                 125
```

```
Ser Met Val Lys Thr Ser Gln Trp Lys Lys Glu Arg Ala Arg Tyr Gly
        130                 135                 140
Val Glu His Thr Tyr Ser Asp Tyr Glu Val Thr Asp Ser Trp Ala Asn
145                     150                 155                 160
Gly Trp His Leu His Arg Asn Met Leu Leu Phe Leu Asp Arg Pro Leu
                165                 170                 175
Ser Asp Asp Glu Leu Lys Ala Phe Glu Asp Ser Met Phe Ser Arg Trp
            180                 185                 190
Ser Ala Gly Val Val Lys Ala Gly Met Asp Ala Pro Leu Arg Glu His
        195                 200                 205
Gly Val Lys Leu Asp Gln Val Ser Thr Trp Gly Gly Asp Ala Ala Lys
    210                 215                 220
Met Ala Thr Tyr Leu Ala Lys Gly Met Ser Gln Glu Leu Thr Gly Ser
225                 230                 235                 240
Ala Thr Lys Thr Ala Ser Lys Gly Ser Tyr Thr Pro Phe Gln Met Leu
                245                 250                 255
Asp Met Leu Ala Asp Gln Ser Asp Ala Gly Glu Asp Met Asp Ala Val
            260                 265                 270
Leu Val Ala Arg Trp Arg Glu Tyr Glu Val Gly Ser Glu Lys Asn Leu Arg
        275                 280                 285
Ser Ser Trp Ser Arg Gly Ala Lys Arg Ala Leu Gly Ile Asp Tyr Ile
    290                 295                 300
Asp Ala Asp Val Arg Arg Glu Met Glu Glu Glu Leu Tyr Lys Leu Ala
305                 310                 315                 320
Gly Leu Glu Ala Pro Glu Arg Val Glu Ser Thr Arg Val Ala Val Ala
                325                 330                 335
Leu Val Lys Pro Asp Asp Trp Lys Leu Ile Gln Ser Asp Phe Ala Val
            340                 345                 350
Arg Gln Tyr Val Leu Asp Cys Val Asp Lys Ala Lys Asp Val Ala Ala
        355                 360                 365
Ala Gln Arg Val Ala Asn Glu Val Leu Ala Ser Leu Gly Val Asp Ser
    370                 375                 380
Thr Pro Cys Met Ile Val Met Asp Asp Val Asp Leu Asp Ala Val Leu
385                 390                 395                 400
Pro Thr His Gly Asp Ala Thr Lys Arg Asp Leu Asn Ala Ala Val Phe
                405                 410                 415
Ala Gly Asn Glu Gln Thr Ile Leu Arg Thr His
            420                 425
```

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<210> 9
<211> 30
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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for
      introducing mutation to pAM330

<400> 9
aaacccgggc tacgtctgat gctttgaatc   30

<210> 10
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for
      introducing mutation to pAM330

<400> 10
tttgatcccc cgttaacgtc aacaacc   27
<210> 11
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for
      introducing mutation to pAM330

<400> 11
ttttcccggg agcttgccac accccgag   28

<210> 12
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for
      introducing mutation to pAM330

<400> 12
ggggtcatc tctggctgaa ttgg   24

<210> 13
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for
     introducing mutation to pAM330

<400> 13
gaggttttca ccgttctgca tgcc   24

<210> 14
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for
     introducing mutation to pAM330

<400> 14
aactcaccgc cctgcaattc aac   23

<210> 15
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for PCR

<400> 15
gcctaccgcg gcaaagaagt ggcag   25

<210> 16
<211> 25

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for PCR

<400> 16
gccttgaact aggggcgctt taagt   25

<210> 17
<211> 4235
<212> DNA
<213> Brevibacterium flavum

<220>
<221> CDS
<222> (1852)..(2364)

<400> 17
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cacgcttgac tggtcgttcc cggattctgg ctgcagttca tggtttccac ggccgcacca 120
tgggttccct cgcgctgact ggccagccag acaagcgtga agcgttcctg ccaatgccaa 180
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acccaacgga tgtggctgct atcttcctcg agccaatcca gggtgaaacg ggcgttgttc 300
cagcacctga aggattcctc aaggcagtgc gcgagctgtg cgatgagtac ggcatcttga 360
tgatcaccga tgaagtccga actggcgttg gccgtaccgg cgatttcttt gcacatcagc 420
acgatggcgt tgttcccgat gtggtgacca tggccaaggg acttggcgc ggtcttccca 480
tcggtgcttg tttggccact ggccgtgcag ctgaattgat gaccccaggc aagcacggca 540
ccactttcgg tggcaaccca gttgcttgtg cagctgccaa ggcagtgctg tctgttgtcg 600
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cggcggacaa cattatccgt ttgaccccgc cgctggtgat caccgacgaa gaaatcgcag 840
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```
gtggaaaacc tcagccctga agaaggccca gcaggcctta agggtaagaa ggctgtgtac 1380
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                                                        Met Ser
                                                          1
```

```
ctt ggc tca acc ccg tca aca ccg gaa aac tta aat ccc gtg act cgc    1905
Leu Gly Ser Thr Pro Ser Thr Pro Glu Asn Leu Asn Pro Val Thr Arg
          5                  10                  15
act gca cgc caa gct ctc att ttg cag att ttg gac aaa caa aaa gtc    1953
Thr Ala Arg Gln Ala Leu Ile Leu Gln Ile Leu Asp Lys Gln Lys Val
      20                  25                  30
acc agc cag gta caa ctg tct gaa ttg ctg ctg gat gaa ggc atc gat    2001
Thr Ser Gln Val Gln Leu Ser Glu Leu Leu Leu Asp Glu Gly Ile Asp
35                  40                  45                  50
atc acc cag gcc acc ttg tcc cgg gat ctc gat gaa ctc ggt gca cgc    2049
Ile Thr Gln Ala Thr Leu Ser Arg Asp Leu Asp Glu Leu Gly Ala Arg
              55                  60                  65
aag gtt cgc ccc gat ggg gga cgc gcc tac tac gcg gtc ggc cca gta    2097
Lys Val Arg Pro Asp Gly Gly Arg Ala Tyr Tyr Ala Val Gly Pro Val
              70                  75                  80
gat agc atc gcc cgc gaa gat ctc cgg ggt ccg tcg gag aag ctg cgc    2145
Asp Ser Ile Ala Arg Glu Asp Leu Arg Gly Pro Ser Glu Lys Leu Arg
              85                  90                  95
cgc atg ctt gat gaa ctg ctg gtt tct aca gat cat tcc ggc aac atc    2193
Arg Met Leu Asp Glu Leu Leu Val Ser Thr Asp His Ser Gly Asn Ile
      100                 105                 110
gcg atg ctg cgc acc ccg ccg gga gct gcc cag tac ctg gca agt ttc    2241
Ala Met Leu Arg Thr Pro Pro Gly Ala Ala Gln Tyr Leu Ala Ser Phe
115                 120                 125                 130
atc gat agg gtg ggg ctg aaa gaa gtc gtt ggc acc atc gct ggc gat    2289
Ile Asp Arg Val Gly Leu Lys Glu Val Val Gly Thr Ile Ala Gly Asp
              135                 140                 145
gac acc gtt ttt gtt ctc gcc cgt gat ccg ctc aca ggt aaa gaa cta    2337
Asp Thr Val Phe Val Leu Ala Arg Asp Pro Leu Thr Gly Lys Glu Leu
              150                 155                 160
```

```
ggt gaa tta ctc agc ggg cgc acc act taaagcgccc ctagttcaag        2384
Gly Glu Leu Leu Ser Gly Arg Thr Thr
        165                 170
gcttgttaat cgcttgttaa tgcaggcagg taaggtataa cccgagtgtt ttttcgagga 2444
ataccaaccc tttcaacaca ataattttct ttaaacatcc ttgctgtcca ccacggctgg 2504
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ggccgtctgc gcgctggtcg ttcccgcaac gaccaggtgg caccctgtt ccgcatgtgg 4184
gtccgcgacg cagtgcgcgca catcgcgctg ggaacaaccg agcttgtcga c            4235
```

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<210> 18
<211> 171
<212> PRT
<213> Brevibacterium flavum

<400> 18
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Met Ser Leu Gly Ser Thr Pro Ser Thr Pro Glu Asn Leu Asn Pro Val
 1               5                   10                  15
Thr Arg Thr Ala Arg Gln Ala Leu Ile Leu Gln Ile Leu Asp Lys Gln
            20                  25                  30
Lys Val Thr Ser Gln Val Gln Leu Ser Glu Leu Leu Leu Asp Glu Gly
        35                  40                  45
Ile Asp Ile Thr Gln Ala Thr Leu Ser Arg Asp Leu Asp Glu Leu Gly
    50                  55                  60
Ala Arg Lys Val Arg Pro Asp Gly Gly Arg Ala Tyr Tyr Ala Val Gly
65                  70                  75                      80
Pro Val Asp Ser Ile Ala Arg Glu Asp Leu Arg Gly Pro Ser Glu Lys
                85                  90                  95
Leu Arg Arg Met Leu Asp Glu Leu Leu Val Ser Thr Asp His Ser Gly
            100                 105                 110
Asn Ile Ala Met Leu Arg Thr Pro Pro Gly Ala Ala Gln Tyr Leu Ala
        115                 120                 125
Ser Phe Ile Asp Arg Val Gly Leu Lys Glu Val Val Gly Thr Ile Ala
    130                 135                 140
Gly Asp Asp Thr Val Phe Val Leu Ala Arg Asp Pro Leu Thr Gly Lys
145                 150                 155                 160
Glu Leu Gly Glu Leu Leu Ser Gly Arg Thr Thr
                165                 170
```

<210> 19
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for PCR

<400> 19
cccgggtttt cttctgcaac tcggg   25

<210> 20
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for PCR

<400> 20
gtcgacaagc tcggttgttc ccagc   25

<210> 21
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for PCR

<400> 21
cccctagttc aaggcttgtt aatc   24

<210> 22
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer for PCR

<400> 22
gtcttacctc ggctggttgg ccagc   25